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LACTOBACILLUS MEDIATED DELIVERY OF THERAPEUTIC PEPTIDES IN THE GASTROINTESTINAL TRACT

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Lactobacillus Mediated Delivery of Therapeutic Peptides in the Gastrointestinal Tract

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To the affliction, I devoted my affection.

实验虐我千百遍，我待实验如初恋。

To my dearest parents

献给我最爱的父母

ABSTRACT

Lactobacilli are normal residents of the human gastrointestinal (GI) tract. Many species belonging to the genera *Lactobacillus* with health beneficial properties have been introduced as probiotics. Genetic engineering of *Lactobacillus* could potentially combine the colonizing ability and probiotic effect with an engineered therapeutic function. The aim of this thesis was to explore the possibility of using lactobacilli as vectors for delivery of peptides in the GI tract for therapy against type 2 diabetes and other immune-related diseases.

GLP-1, an incretin hormone that can stimulate insulin secretion, is used in the treatment of type 2 diabetes. In paper I, we designed and expressed the pentameric GLP-1 (trypsin cleavable oligomers) both in a secreted form and anchored on the surface of *L. paracasei* BL23. The pentameric GLP-1 retains its bioactivity both *in vitro* and in the intestine of diabetic rat following digestion by intestinal trypsin. When given by gavage to diabetic rats, the non-expressor *Lactobacillus* showed significant anti-diabetic effect but GLP-1 expression did not provide an additional insulinotropic effect possibly due to the low levels produced. These results indicate that *L. paracasei* BL23 itself might be used as an alternative treatment method for type 2 diabetes, but further work is needed to increase the expression level of GLP-1 by *Lactobacillus*.

Interleukin-22 (IL-22) plays a prominent role in epithelial regeneration and protecting intestinal stem cells from immune-mediated tissue damage. In paper II, *Lactobacillus* strains were constructed for delivery of IL-22 as a new therapeutic strategy for acute graft-versus-host disease (GVHD) in the GI tract. The secretion and surface anchoring of mouse IL-22 by *L. paracasei* BL23 was demonstrated and the biological activity of IL-22 produced by *Lactobacillus* was shown *in vitro.*

In paper III, we developed a co-expression vector to produce two rotavirus-specific VHH antibody fragments in *Lactobacillus*. Both antibody fragments (ARP1 and ARP3) were expressed in secreted and surface anchored forms and their ability to bind to various rotavirus serotypes was demonstrated *in vitro*. This vector was then used in paper IV, to simultaneously produce both secreted Interleukin-21 (IL-21) and anchored CD40 ligand (CD40L) on the surface of lactobacilli. IL-21/CD40L based stimulation may be a potential therapy for IgA deficient patients. In this paper, IL-21(or hybrid IL-21/4) and CD40L were expressed individually, or co-expressed in *L. paracasei* BL23. We showed that *Lactobacillus* expressing IL-21, IL-21/4 and CD40L individually can induce IgA secretion in PBMCs from healthy donors.

This work provides the basis for the use of genetically modified lactobacilli for delivery of therapeutic peptides in the GI tact. The delivery strategies could be further developed to facilitate easy and inexpensive access to other peptide drugs.

LIST OF SCIENTIFIC PAPERS

I. **Oral delivery of pentameric glucagon-like peptide-1 by recombinant *Lactobacillus* in diabetic rats.**
Lin Y, Krogh-Andersen K, Pelletier J, Marcotte H, Östenson C-G, Hammarström L
PLoS ONE. 2016 11(9): e0162733.

II. **Oral delivery of *Lactobacillus* that secretes bioactive interleukin-22.**
Lin Y, Hammarström L, Marcotte H.
Submitted.

III. **Expression of anti-rotavirus proteins (llama VHH antibody fragments) in *Lactobacillus*: Development and functionality of vectors containing two expression cassettes in tandem.**
Günaydın G, Álvarez B, **Lin Y**, Hammarström L, Marcotte H.
PLoS ONE. 2014 9(4): e96409.

IV. ***Lactobacillus* delivery of interleukin-21 and CD40 ligand for treatment of selective IgA deficiency.**
Lin Y, Borte S, Appelberg S, Marcotte H, Hammarström L.
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

APF	Aggregation promoting factor
APC	Antigen-presenting cells
CFU	Colony forming unit
CSR	Class-switch recombination
DNA	Deoxyribonucleic acid
DPP-4	Dipeptidyl peptidase-4
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
FITC	Fluorescein isothiocyanate
GI tract	Gastrointestinal tract
GK rat	Goto-Kakizaki rat
GLP-1	Glucagon-like peptide-1
GRAS	Generally regarded as safe
GVHD	Graft-versus-host diseases
HBC	Hyperimmune bovine colostrum
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
Ig	Immunoglobulin
IgAD	IgA deficiency
IPGTT	Intraperitoneal glucose tolerance test
MHC	Major histocompatibility complex
MRS	Mann Rogosa Sharpe lactobacilli media
NICE	Nisin controlled gene expression
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PID	Primary immunodeficiency diseases
SCFAs	Short-chain fatty acids
ScFv	Single chain fragment variable
TNF	Tumor necrosis factor
VHH	Variable fragment of heavy chain antibody

1 INTRODUCTION

1.1 *LACTOBACILLUS*

Lactobacilli are rod-shaped, Gram-positive lactic acid bacteria that are classified as "generally recognized as safe" (GRAS) [1]. Lactic acid bacteria are an order of bacteria exhibiting an enormous capacity to degrade different carbohydrates and produce lactic acid as their main end product [2]. They have been historically used in food fermentation and preservation as acidification inhibits the growth of spoilage agents. Lactobacilli are normal residents of the gastrointestinal (GI) tract of animals and humans, as shown in Figure 1.1. In total, 17 *Lactobacillus* species can be detected within the human GI tract, although some of them are only transient colonizers [3, 4]. The highest density of lactobacilli is found in the ileum (up to 10^8/ml) and in the colon (10^9/ml). Among them, the dominant colonizers are *L. gasseri*, *L. crispatus*, *L. reuteri*, *L. casei* and *L. salivarius* [2, 5].

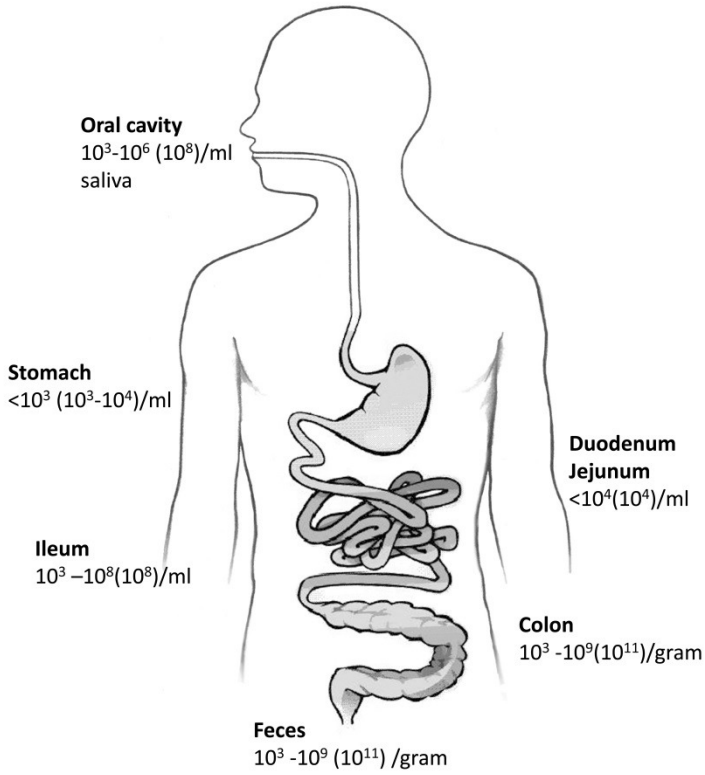

Figure 1 The distribution of lactobacilli in the oral cavity and the gastrointestinal tract. The number in bracket shows the total bacterial load in this organ. Data extracted from literature [4, 6-8].

Probiotics are live bacteria isolated from human or food product defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" [9]. *Lactobacillus* is the best-studied probiotic genus, and may modulate metabolic activities of the gut microbiota by competing with and displacing pathogens, produce antimicrobial agents (such as bacteriocins and short-chain fatty acids (SCFAs)), enhance barrier function, induce anti-inflammatory responses or modulate immune response [2, 10]. Several studies have demonstrated a beneficial effect of *Lactobacillus* consumption for

mitigation of gastrointestinal (GI) diseases [11-13] such as irritable bowel syndrome (IBS), Inflammatory bowel disease (IBD) and rotavirus infection. Some strains of *Lactobacillus* have even been shown to exert an anti-diabetic effect in animal models [14-16] and humans [17].

1.2 *LACTOBACILLUS* AS A DELIVERY SYSTEM

Bioengineering of the probiotic *Lactobacillus* strains could potentially combine the colonizing ability and probiotic effect with an engineered therapeutic function. Selected probiotic *Lactobacillus* strains could colonize the GI tract for weeks [18]. Orally administered engineered *Lactobacillus* strains could survive the gastrointestinal passage and transiently colonize the GI tract where they could constitutively produce therapeutic molecules, reducing their exposure to gastric acid, bile and digestive enzymes. This would provide a continuous supply of biologically active peptides, which, could directly interact with receptors in the gut or be absorbed through the intestinal epithelium [19].

1.2.1 *Lactobacillus* expression systems

Therapeutic peptides expressed in *Lactobacillus* are normally designed to be anchored on the cell wall surface or secreted into the culture medium. Therefore, a strong promoter and a functional signal peptide fused to the peptide-encoding genes are needed for their expression. To maximize the expression level, inducible promoters have been employed in order to reduce the growth pressure caused by "toxic" peptides. Most popular systems, relying on quorum sensing mechanism to drive gene expression, include the NICE (nisin-controlled gene expression) system from *Lactococcus lactis* and the sakacin-based expression system from *Lactobacillus sakei* [20, 21]. However, inducible systems are less suitable for *in situ* production in the GI tract, and a constitutive active promoter enabling stable expression level is required for such applications [22]. Signal peptides can also affect protein expression and secretion, and many studies have explored different signal peptides for protein production in *Lactobacillus* [23-25]. The prediction of the signal peptide cleavage site is possible [26], but there are no specific rules in the selection of signal peptide as it may depend on the fused peptide and the *Lactobacillus* strains [23, 27].

Besides the secreted peptides, cell wall anchoring can be achieved by fusing the peptide gene with a selected protein binding domain from lactobacilli. The peptide could attach to the cell surface either covalently via the LPXTG motif or noncovalently through hydrophobic or ionic interactions [28, 29]. Covalent anchoring is mediated through a sortase-dependent C-terminal anchoring domain which contains a cleavage site, the conserved LPXTG motif, located in the C-terminal region of the protein [28]. After secretion, the sortase cleaves the LPXTG motif between the threonine and glycine and links the protein covalently to the cell wall peptidoglycan [29].

The expression system developed in our group is regulated by a constitutive promoter of the aggregation promoting factor (*apf*) gene from *Lactobacillus crispatus* M247 and secretion is mediated by the signal peptide of the *apf* gene. For covalent surface anchoring on the cell

wall, the gene of interest is fused to the sequence encoding the last 231 amino acids of the proteinase P surface protein (PrtP) (containing a C-terminal LPXTG motif) from *L. paracasei* BL23 [30]. Most expression systems in lactobacilli are plasmid-based vectors that contain antibiotic markers. For therapeutic use *in vivo*, food-grade chromosomal integrated expression systems which are antibiotic-free have been developed [30-33]. The integrative expression systems showed a stable expression level, however, a reduced expression level might be observed due to that each bacterium contains one copy of the chromosomally integrated gene but multi-copies of the plasmid carried gene.

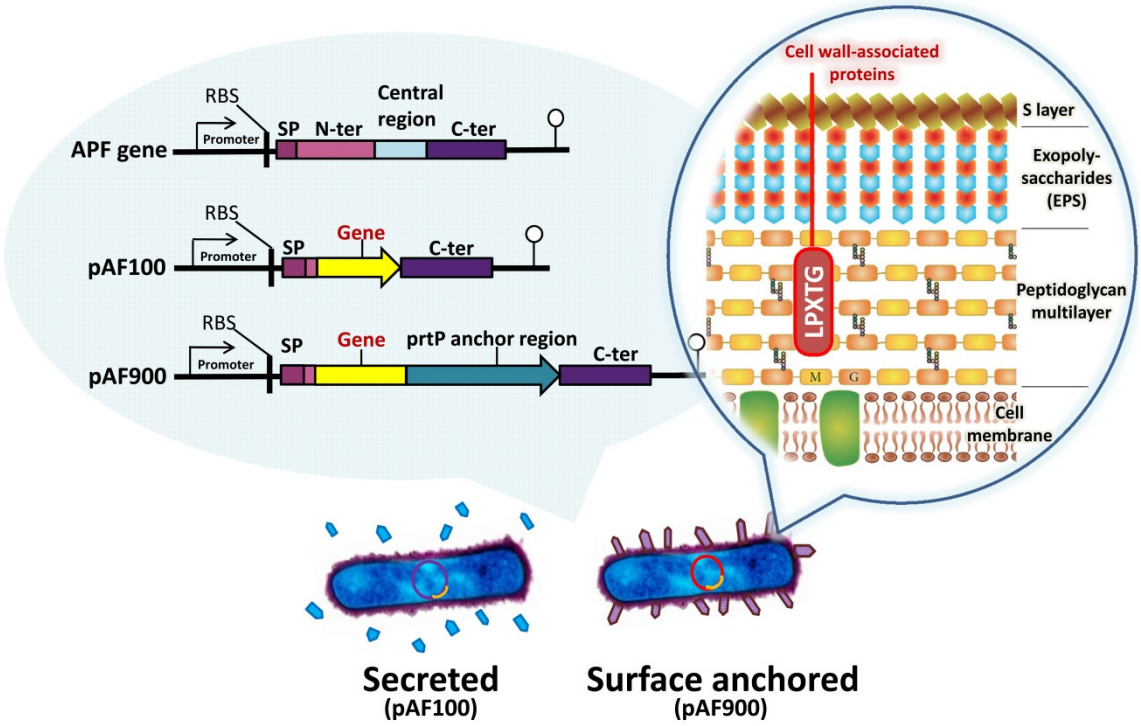

Figure 2 Expression cassettes of the pAF plasmid series for secreted and surface display of protein in lactobacilli. SP, signal peptide; RBS, ribosomal binding site; C-ter, C-terminal part of APF gene; translation stop codon (indicated with an arrowhead) and the transcription terminator (indicated with a lollipop). Cell surface of lactobacilli with a schematic representation of cell-wall associated proteins (the figure was adapted from [28]). The proteins are attached covalently to the cell wall through a LPXTG sorting motif.

1.2.2 Delivery of therapeutic molecules

Lactic acid bacteria, especially *Lactococcus lactis* and *Lactobacillus*, have become popular vehicles to deliver prophylactic and therapeutic molecules to the mucosa. In the beginning, the work mainly focused on delivering antigens for mucosal vaccination since they can elicit antigen-specific secretory IgA responses at the mucosal surface [1]. The initial work on delivery of therapeutic molecules was carried out in *L. lactis* [34], a strain widely used in the dairy industry and with high transformation efficiency. One of the most promising studies was the use of IL-10-secreting *L. lactis* to treat inflammatory bowel disease (IBD) in mouse models [35] and humans [36]. Lactobacilli are natural inhabitants of the human GI tract, and

transient colonization of the gut might provide prolonged delivery of peptides, an advantage compared to *L. lactis*.

Lactobacilli have been used for delivery of passive immunity in the oro-gastrointestinal tract. Lactobacilli producing ScFv or VHH antibody fragments were shown to confer protection against *Streptoccus mutans* [37, 38], rotavirus [39, 40], *Clostridium difficile* toxin B [41] and anthrax toxin [42] in rodent models of infection. The antibody fragments produced and locally delivered by lactobacilli at the mucosal sites could provide an efficient therapy at a low cost [43]. Single chain variable fragment (ScFv) is about 30 kDa and consists of variable light (VL) and heavy (VH) regions joined together by a flexible peptide linker. The variable domain of llama heavy chain antibody (VHH, or nanobody) is about 15 kDa. VHH is a stable single-domain molecule with superior solubility, more acid and heat resistant, and binding with similar affinities as conventional antibodies [44]. These traits made it markedly easier to express in a functional recombinant form in lactobacilli. *Lactobacillus* producing surface-anchored llama VHH antibody fragment (named ARP1) was shown to bind to rotavirus *in vitro* and protect mouse pups against rotavirus-induced diarrhea [39, 40]. Furthermore, *Lactobacillus* producing surface anchored VHH dimer, consisting of ARP1 and ARP3 antibody fragments directed against VP6 proteins, have an increased therapeutic effect in a mouse model of rotavirus infection compared to monovalent VHH [40], probably because ARP1 and ARP3 fragments could target multiple epitopes and therefore acting synergistically against the virus [45].

Until now, *Lactobacillus* have been used to deliver different therapeutic molecules like antigens [46-50], antibody fragments [37, 39, 40, 42], cytokines [51-53], enzymes [54] and DNA molecules [55]. This thesis focused on delivery of therapeutic peptide (incretin peptide and cytokines) by *Lactobacillus* for treatment of metabolic and immunological disorders.

1.3 GLUCAGON-LIKE PEPTIDE-1 AND TYPE 2 DIABETES

1.3.1 Type 2 diabetes

Type 2 diabetes, formally called non-insulin-dependent diabetes mellitus, is a metabolic disorder characterized by high blood glucose levels due to insulin resistance and relative insulin deficiency [56]. The World Health Organization estimates that 422 million people in the world had diabetes in 2014, of which about 90% was type 2 diabetes. The prevalence of diabetes is steadily increasing everywhere, most markedly in the middle-income countries [57]. Type 2 diabetes was previously seen only in adults, but it is now increasing rapidly among children and adolescents. Treatment of type 2 diabetes involves healthy diet, regular physical exercise and oral medication (sulfonylureas, biguanides and thiazolidinediones)[58], or insulin therapy.

1.3.2 Glucagon-like peptide-1 (GLP-1)

Glucagon-like peptide-1 (GLP-1) is a proglucagon-derived peptide produced by intestinal endocrine L cells in response to nutrient ingestion. GLP-1 is an incretin hormone that can

stimulate insulin secretion from the pancreas in a glucose-dependent manner, reduce glucagon secretion and slow down gastric emptying [59]. Exogenously supplied GLP-1 can normalize glucose levels following subcutaneous injections in type 2 diabetic patients [60, 61]. GLP-1 can also reduce food intake and body weight in obese patients with type 2 diabetes [62, 63]. Furthermore, GLP-1-induced stimulation of insulin secretion is strictly glucose-dependent and does not cause hypoglycemia, a severe side effect of some medications presently used for treatment of diabetes [64]. Therefore, GLP-1 and its analogues provide an attractive option for treatment of type 2 diabetes.

GLP-1 is a short peptide that has a highly conserved amino acid sequence in mammals [65]. The inactive full-length form of GLP-1 (1-37) is processed into two active circulating forms: GLP-1 (7–37) and GLP-1 (7-36) amide, with the latter being the most abundant form found in blood. Both forms of GLP-1 are susceptible to dipeptidyl peptidase-4 (DPP-4) -mediated degradation and inactivation. GLP-1 has a very short half-life (< 2 minutes) and its concentration returns to baseline within 90 min after subcutaneous injection [66], making it difficult to administer native GLP-1 systemically.

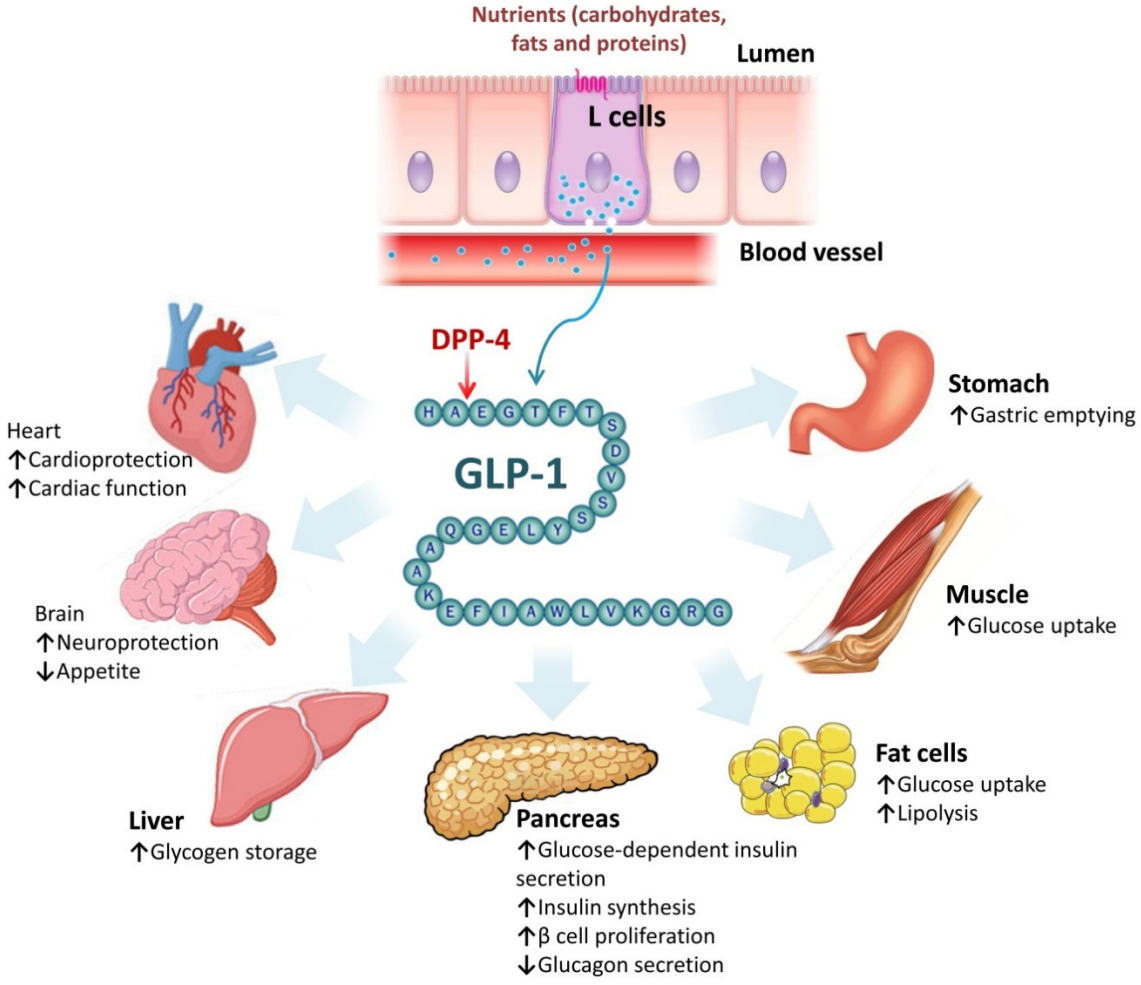

Figure 3 Physiology of GLP-1 secretion and actions in peripheral organs and tissues. GLP-1 is produced by intestinal L-cells upon nutrients stimulation, and secreted into intestinal small blood vessels. GLP-1 stimulates insulin biosynthesis and secretion in the pancreas through binding to their distinct receptors on β cells. GLP-1 acts directly on the endocrine pancreas,

stomach, heart and brain, whereas actions on muscle and liver are indirect. Figure adapted from [67, 68].

1.3.3 GLP-1 based diabetes therapies

An alternative treatment involving the GLP-1 pathway is the oral administration of DPP-4 inhibitors which results in a physiological increase in GLP-1 levels [69, 70]. However, most recent research has focused on developing long-acting GLP-1 receptor agonists. GLP-1-Gly8, a 31 amino acid peptide of GLP-1 (7–37) with the alanine in position 8 changed into glycine (Gly), prolongs the half-life of the peptide by altering the cleavage site for the DPP-4 [71]. Approved GLP-1 analogues, including Exenatide (Byetta®) and Liraglutide (Victoza®), can prolong the half-life of the peptide to hours [72, 73]. Once-weekly preparation of a GLP-1 analogue (Albiglutide, Dulaglutide) was also available for therapy [74].

GLP-1 analogues are currently only administered through the subcutaneous route due to degradation by gastrointestinal enzymes [69]. Since GLP-1 is produced predominantly from the ileum and colon [75], it is not equally distributed throughout the systemic circulation and the highest concentrations are found in the splanchnic blood [76]. Therefore, the current subcutaneous injection does not mimic the physiological secretion of GLP-1 [76, 77]. In contrast, oral delivery of GLP-1, followed by uptake in the intestinal mucosa, would appear more likely to mimic the physiological route while providing a comfortable and convenient drug delivery method for patients. A few efforts have previously been made to solve the oral delivery problems of GLP-1 by adding functional groups to facilitate absorption [78], by PEGylation or encapsulating GLP-1 into nanoparticles to protect the peptides from degradation in the GI tract [79-81].

1.3.4 Advantages of using lactobacilli for delivery of GLP-1

Engineered *Lactobacillus* could be used to produce and locally deliver GLP-1 peptide in the GI tract, where natural GLP-1 is produced. *Lactobacillus* could continuously produce biologically active GLP-1, reducing their exposure to gastric acid, bile and digestive enzymes.

As one of the best studied probiotic species, some strains of *Lactobacillus* have been shown to exert an anti-diabetic effect in rodents by lowering the blood glucose level [14, 15, 82-84]. A recent report also showed that *L. reuteri* improves incretin and insulin secretion in glucose-tolerant humans [17]. According to Panwar *et al.* [16], different *Lactobacillus* strains, including *L. rhamnosus* GG*, L. casei, L. acidophilus, L. plantarum* and *L. reuteri,* have been shown to either lower plasma glucose levels or to improve insulin resistance in diabetic mouse or rat models. In addition, a recent paper demonstrated that feeding a *L. gasseri*-delivered "receptor-inactive" full-length GLP-1 (1-37) in a type 1 diabetic rat model for a period of 90 days, could reprogram intestinal epithelial cells into "glucose-responsive insulin-secreting cells" [85], suggesting that lactobacilli will be suitable candidates for *in vivo* "receptor-active" GLP-1 (7-37) peptide delivery. Bioengineering of the probiotic

Lactobacillus strains could potentially combine the colonizing ability and anti-diabetic effect of lactobacilli with an engineered GLP-1 therapeutic function.

1.4 INTERLEUKIN-22 AND GRAFT-VERSUS-HOST DISEASE

1.4.1 Graft-versus-host disease

Graft-versus-host disease (GVHD) is a frequent and challenging complication that occurs after allogeneic stem cell transplantation in which the newly transplanted immune cells attack the tissues of the recipient [86, 87]. Clinical GVHD carries approximately a 50% mortality rate, which contributes to most transplant-related morbidity and mortality [88]. GVHD occurs in acute and chronic forms. Following hematopoietic stem-cell transplantation, acute GVHD develops within 100 days when graft-derived T cells are activated against antigens from the recipient, involving mainly the gastrointestinal (GI) tract, the skin, and the liver [89, 90]. Acute GVHD of the GI tract is often severe and is a significant cause of transplant-associated morbidity [90]. Chronic GVHD describes a more diverse syndrome and normally occurs after 100 days post transplantation [89].

The incidence of acute GVHD ranges between 26% and 32% in recipients of HLA matched sibling donor grafts, and from 42% to 52% in recipients of HLA matched unrelated donor grafts [91]. The minor histocompatibility antigens, which are small peptides derived from polymorphic proteins presented by MHC molecules, are responsible for acute GVHD in these HLA matched transplantation [92]. The standard treatment for GVHD is immunosuppressive therapy with corticosteroids (such as prednisone), while side effects like increased risk of dangerous infections could happen to the patients whose immune systems are already fragile after transplantation [93]. Therefore, it is important to further study the pathogenesis of GVHD and to develop new therapeutic options.

1.4.2 Interleukin-22

Interleukin-22 (IL-22) is a member of the IL-10 family of cytokines, expressed predominantly by subsets of innate lymphoid cells (ILCs) and activated T cells, including T helper 1 (TH1) cells, TH17 cells and TH22 cells [94, 95]. The mature, secreted form of human IL-22 consists of 146 amino acids [95], which shares a 79% amino acid sequence identity with mouse IL-22 and a 25% identity with human IL-10. IL-22 can be recognized by a heterodimeric receptor complex that consists of two transmembrane subunits: IL-22R1 and IL-10R2 [96]. The binding of IL-22 to its receptor activates the JAK/STAT and MAPK signaling pathways [97, 98], resulting in gene expression or repression. Since the IL-10R2 is shared by five cytokines (IL-10, IL-22, IL-26, IL-28, and IL-29) and is widely expressed in most cells [99], the expression of the IL-22R1 determines whether a cell is the target of IL-22. The lack of expression of IL-22R1 in all immune cells indicates that IL-22 does not directly regulate the function of the immune system [100]. The targets of IL-22 are mostly non-hematopoietic epithelial and stromal cells in the intestines, lung, liver, pancreas, thymus, kidney and skin [94, 95, 101].

IL-22 can induce the production of antibacterial peptides from epithelial cells and selected chemokines in selected tissues, where it can promote epithelial cell survival and proliferation, play an important role in tissue regeneration and protect against damage induced by chronic inflammation [94, 102-104]. Additionally, IL-22 can directly stimulate goblet cells to secrete mucins, which forms the protecting mucus layer [105]. It can also protect intestinal epithelium stem cells from immune-mediated tissue damage and increase their proliferation [90]. Local IL-22 gene delivery has been shown to lead to rapid attenuation of intestinal inflammation in the colon in a Th2-mediated chronic colitis mouse model [106]. Therefore, IL-22 is an attractive and promising target for inflammatory bowel disease (IBD) therapy [107]. It might also have beneficial effects in alcoholic liver disease, pancreatic damage and GVHD [94].

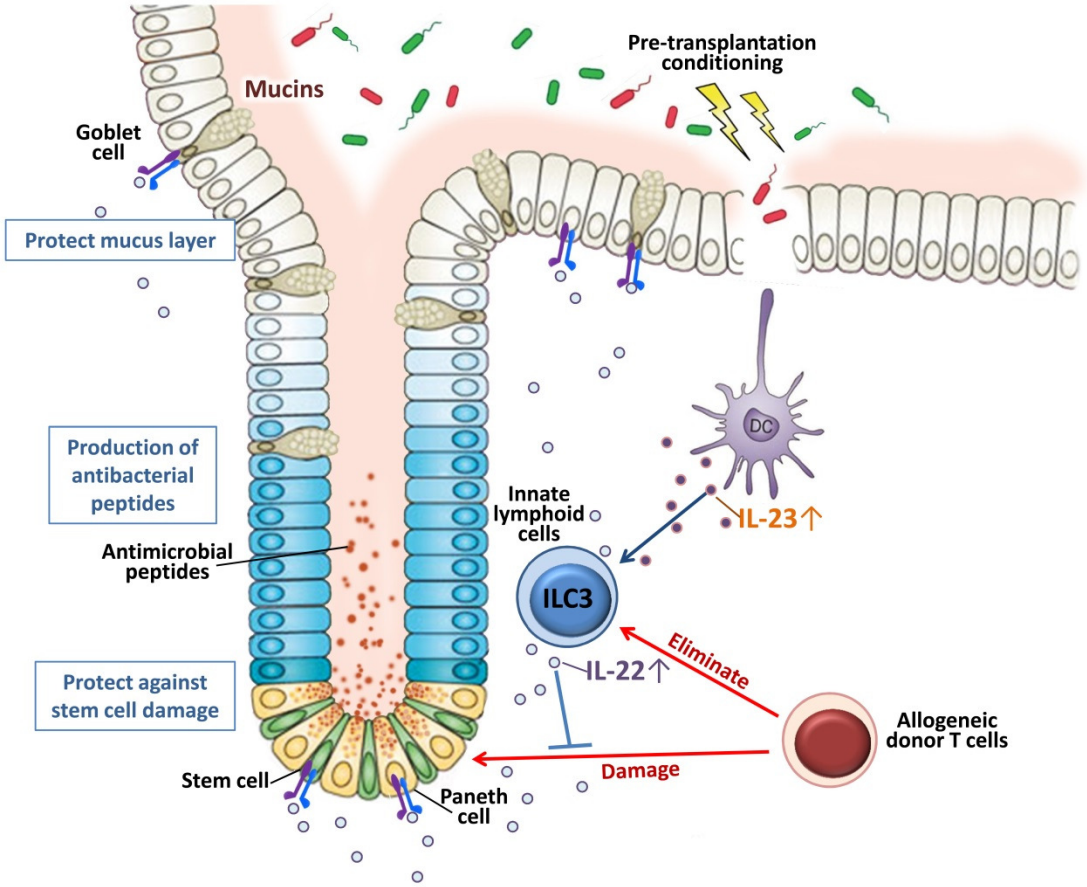

Figure 4 IL-22 protects intestinal stem cells from GVHD-mediated tissue damage. Pre-transplantation conditioning induces IL-23 production from the GI tract. IL-23 stimulates ILC3 to produce IL-22, which then protects the intestinal stem cell compartment and promotes epithelial recovery from tissue damage. During GVHD, Allogeneic donor T cells can eliminate ILC3 and damage the intestinal stem cells. Figure adapted from [90, 94].

1.4.3 IL-22 in the treatment of acute GVHD

Rorγt^+ IL-23-responsive innate lymphoid cells, which are present in the gut cryptopatches, are a major source of IL-22 [108]. Studies have shown that after allogeneic hematopoietic cell transplantation, donor-derived IL-22 aggravates systemic inflammation and increases mortality of the recipients [109, 110]. IL-22 deficiency in donor T cells reduced mortality and

severity of acute GVHD for the recipients after transplantation [90, 109]. In contrast, IL-22 deficiency in recipient mice led to increased intestinal GVHD pathology and accelerated mortality, indicating that supplement of IL-22 for the recipient may have a therapeutic potential in GVHD [90]. Moreover, recombinant mouse IL-22 treatment via intraperitoneal injection enhanced the recovery of intestinal stem cells (ISCs), increased epithelial regeneration and reduced intestinal pathology and mortality from GVHD in allogeneic bone marrow transplanted mice [111, 112].

Due to the lack of IL-22 receptor on immune cells, IL-22 could potentially be used as a relatively safe molecule to treat GVHD without causing any immune-related side effects [94]. Administration of recombinant IL-22 to patients with GVHD could support the regeneration of epithelial cells without triggering inflammation, which would aggravate the disease. Meanwhile, the IL-22 receptor is predominantly expressed on mucosal epithelial cells. An oral formulation could thus be a potential way to deliver IL-22 directly to the intestine and interact with receptors on the basolateral surface of epithelial cells.

1.4.4 Advantages of using lactobacilli for delivery of IL-22

The gut microbiota itself is also known to have an impact on GVHD [113, 114]. Targeting anaerobic bacteria and introducing potentially beneficial bacteria could reduce the GVHD score [115, 116]. Re-introduction of a mouse commensal, *L. johnsonii*, alleviates GVHD lethality and pathology in a mouse model probably due to the prevention of *Enterococcus* expansion which may otherwise exacerbate GVHD-associated intestinal inflammation [117]. Genetically engineered *Lactobacillus* could be used to deliver IL-22 directly to the intestinal GVHD lesions, providing a continuous supply of bioactive IL-22 during the progression of the disease.

1.5 INTERLEUKIN-21, CD40 LIGAND AND IGA DEFICIENCY

1.5.1 IgA deficiency

Selective IgA deficiency (IgAD) is defined by a serum IgA level equal to or below 0.07 g/l with normal serum IgM and IgG levels in individuals of 4 years of age or older [118]. IgAD is the most prevalent primary immunodeficiency diseases (PID) with an estimated frequency of 1/600 in the Caucasian population [119]. Most IgAD patients are asymptomatic but approximately one third show a significantly increased proneness to recurrent respiratory and gastrointestinal infections and an increased prevalence of allergic diseases and autoimmune disorders as compared to gender- and age-matched controls [119-122]. IgA is the most abundant immunoglobulin class in the body and normally provides immunologic protection at the mucosal membranes including the gastrointestinal and respiratory tract [123, 124]. IgA plays an important role in mucosal immunity in our defense against extracellular pathogens and maintains intestinal homeostasis [123, 125, 126]. IgAD patients have lost the first barrier against pathogens and the capability to control the intestinal microbiota; therefore, they are susceptible to gastrointestinal infections mainly caused by *Giardia lamblia*, *Campylobacter jejuni*, and *Salmonella* spp or lead to overgrowth of commensal bacteria like *Clostridium*

difficile [127, 128]. IgAD is also associated with an increased frequency of celiac disease, Crohn's disease and ulcerative colitis [120].

There is no specific treatment for IgAD patients but sinopulmonary or GI tract infections with bacteria in IgAD patients are usually treated with antibiotics. Prolonged antibiotic treatment may be inevitable and may thus lead to the development of antibiotic-resistant bacteria and other adverse physiological side effects [129]. The commonly recommended treatment for PID disorders is immunoglobulin G (with only trace amounts of IgA) replacement (IVIG), which has been suggested to alleviate symptoms [130]. This treatment is not routinely available in many countries due to its high cost. It may also lead to anaphylactic reactions due to high titers of anti-IgA antibodies present in some IgAD patients [131, 132].

IgAD is a heterogeneous disorder and the major barrier in the development of a therapy for IgAD is the incomplete understanding of the etiology of this disorder. The phenotypic feature of IgAD is a defect in B lymphocyte maturation and subsequent impairment of IgA production [133]. Hereditary genetic defects are associated with IgAD where an association with both MHC (major histocompatibility complex) and non-MHC genes has been reported [119, 134]. The defects in IgA production may result from either a T or a B cell defect, where abnormal cytokine production is suggested to play a role [135, 136]. Previous studies have shown induction of IgA secretion using different models with different stimulators including IL-4, IL-10, IL-21, TGF-β, APRIL and BAFF [125, 137-140]. Among them, the combination of CD40L and IL-21 induced the most robust IgA production in peripheral blood mononuclear cell (PBMC) from healthy donor, and this effect can be enhanced by IL-4 [140].

1.5.2 IL-21 and IL-4

Human interleukin-21 (IL-21) is a cytokine of the γc family expressed by activated $CD4^+$ T cells and NKT cells [141]. IL-21 signals via a heterodimeric receptor complex consisting of the IL-21 receptor (IL-21R) and the γ-chain (γc), the latter being shared by IL-2, IL-4, IL-7, IL-9, and IL-15 [142, 143]. The expression of IL-21 receptor has been detected on B cells, $CD4^+$ T and $CD8^+$ T cells, NK cells, dendritic cells, macrophages and keratinocytes, suggesting that IL-21 has a pleiotropic action on a wide range of cell types [144]. IL-21 has potent regulatory effect on the immune system including promotion of activation, proliferation and differentiation of human B cells [145]. It induces secretion of IgA, IgG, and IgM from all subsets of mature B cells [146]. Recently, patients with PID caused by either IL-21 or IL-21R gene mutations have been described, indicating a critical role of IL-21 in host defense [147-150].

Interleukin-4 (IL-4) is a pleiotropic cytokine mainly expressed by Th2 lymphocytes, basophils, and mast cells [151, 152]. IL-4 is also a member of the γ-chain receptor cytokine family whose receptor complex comprises an IL-4Rα and the common γ-chain shared with IL-21 and other cytokines [153]. IL-4Rα has strong homology to IL-21R and previous work suggested that both IL-4 and IL-21 hold a conserved capability to bind to IL-21R and IL-4Rα [154]. IL-4 was shown to have capacity in B cell growth and differentiation. IL-4 enhances B

cell proliferation induced by CD40L [155], induces class-switch recombination (CSR), and promotes naïve B cells to switch to IgG_1, IgG_4 and IgE production [156-159].

The IL-21 induced class switching and plasma cell formation could be modulated or complemented by additional cytokines, such as IL-4 [155]. IL-4 increased the amount of IgG secreted by naïve B-cell precursors that had been stimulated with IL-21[160, 161]. The IL-21 alone promoted the induction of IgG_3^+ B cells, while the combination of IL-21 and IL-4 favored the generation of IgG_1^+ switched B cells.

1.5.3 CD40L

CD40 ligand (CD40L) is a 261 amino acid transmembrane protein of the tumor necrosis factor (TNF) family, expressed as either membrane-bound or soluble form by activated $CD4^+$ T cells and B cells [162-164]. It binds to CD40 on antigen-presenting cells (APC), which leads to different effects depending on the target cell type. Individuals with CD40 signaling defects suffer from a hyper-IgM symptom with little or no IgA production [165]. CD40L itself has a minimal effect on antibody production by B cells, but different isotypes of antibody can be induced in the presence of different cytokines [166]. Like other TNF family members, the membrane-bound homotrimeric CD40L has the highest bioactivity, while the soluble dimeric and monomeric forms have the lowest activity [167].

Since IL-21 and CD40L signaling is involved in B cell differentiation and antibody class switch recombination [165, 168-171], IL-21 could enhance the proliferation of CD40L-stimulated human B cells. A combination of IL-21 and CD40L induces a strong proliferative response in memory B cells [161], and also induces naïve B cells to enter cell division and isotype switching rapidly [172]. B cells further differentiate into “plasma-like cells” capable of secreting all major immunoglobulin isotypes, predominantly IgG_3 and IgG_1, and to a lesser extent IgA_1 [155].

1.5.4 Treatment of IgAD with IL-21 and CD40L

In vitro stimulation with recombinant CD40L and IL-21 induces IgG or IgA production efficiently in B cells from healthy donors and, to a lesser extent, IgA deficient patients [140]. In combination with IL-2 or IL-4, CD40L and IL-21 can lead to an even stronger IgG and IgA production [140]. This suggests that IL-21/CD40L stimulation may be a potential therapy for patients with IgA deficiency. Furthermore, since IL-21 and IL-4 are able to bind to both IL-21R and IL-4R in an interchangeable manner, a hybrid cytokine which fuses elements of IL-21 and IL-4 cytokines might serve as a more efficient therapeutic agent for IgAD [154].

Intravenously administered human recombinant IL-21 was previously shown to be safe in clinical trials to treat metastatic melanoma and renal cell carcinoma [143, 173, 174]. However, oral delivery via a suitable carrier could improve the patient compliance or efficacy of the cytokines and induce local production of IgA at the mucosal surface where it is most needed.

1.5.5 Advantages of using lactobacilli for delivery of IL-21 and CD40L

Lactic acid bacteria, such as *Bifidobacterium* or *Lactobacillus,* are able to modulate mucosal and systemic immune responses [175-177]. Heat-killed *L. rhamnosus* GG can enhance mucosal IgA production [178], whereas *L. johnsonii* causes increased IgA production in mouse Peyer's patches [179]. The mechanism by which *Lactobacillus* induce IgA production remains unclear, but could be attributed to the surface anchored or secreted proteins [180]. The whole bacteria or derived proteins could be taken by transcytosis through the microfold cell (M cell) in the Peyer's patches, or captured by dendritic cells through extension of dendrites in the gut lumen. IgA class switching in Peyer's patches could also be induced through an alternative T-cell dependent pathway which relies on the TLR signaling by *Lactobacillus* [168, 181].

Lactobacillus, thus appears a good candidate to continuously deliver recombinant IL-21, IL-4 and CD40L that induce IgA production by B lymphocytes at mucosal sites in IgAD patients.

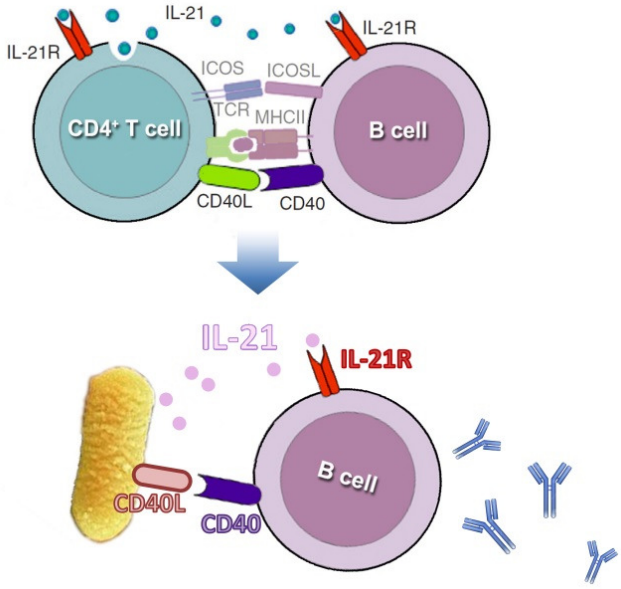

Figure 5 *Lactobacillus* expressing surface anchored CD40L and secreted IL-21 could mimic $CD4^+$ T cells to stimulate B cells.

2 AIMS

2.1 GENERAL AIM

The aim of this thesis was to explore the possibility of using lactobacilli as vectors for delivery of peptides in the gastrointestinal tract for therapy against type 2 diabetes and other immune-related diseases.

2.2 SPECIFIC AIMS

Paper I: To optimize the expression of stabilized GLP-1 analogues in lactobacilli and test the bioactivity of the modified lactobacilli *in vitro* and in the diabetic GK rat model.

Paper II: To construct *Lactobacillus* strains for delivery of IL-22 directly to the intestinal mucosa as a new therapeutic strategy for acute gastrointestinal GVHD.

Paper III: To develop a co-expression vector containing two expression cassettes in tandem to produce two rotavirus-specific VHH antibody fragments (ARP1 and ARP3) in lactobacilli in order to increase therapeutic efficacy. Moreover, this vector can be used to co-express other peptides for therapeutic purpose.

Paper IV: To construct IL-21 and CD40L expressing *Lactobacillus* strains to induce immunoglobulin production in PBMCs from patients with IgAD.

3 MATERIALS AND METHODS

3.1 BACTERIAL STRAINS AND GROWTH CONDITIONS

L. paracasei BL23 (previously named *L. casei* ATCC 393 pLZ15$^-$) [182, 183] was a gift from Dr. Peter Pouwels (TNO Nutrition and Food Research Institute, Netherlands). Lactobacilli were inoculated in liquid MRS medium (Difco, Sparks, MD) from the overnight culture and grown statically to OD_{600} = 1.0 (around 2×10^8 cfu/ml) or anaerobically on MRS-agar plates at 37°C. *E. coli* DH5α (Invitrogen, Carlsbad, CA) was used as a general cloning host and grown in LB broth in an orbital shaker or on LB-agar plates at 37°C. Antibiotics were added when required: 5 μg/ml erythromycin or chloramphenicol for *L. paracasei* BL23, and 300 μg/ml erythromycin or 100 μg/ml ampicillin for *E. coli*.

3.2 SYNTHESIS OF GENES AND PEPTIDES

All the genes and peptides used in this thesis were synthesized by GenScript Corporation (Piscataway, NJ).

In paper I, the wt (wild type) 5×GLP-1 and trp (trypsin stabilized) 5×GLP-1 genes were produced as synthetic genes and codons optimized for expression in *L. paracasei*. The synthetic genes were flanked by an upstream *Nco*I and a downstream *Not*I restriction site for the subsequent cloning process.

In paper II, the mouse IL-22 gene was synthesized with codons optimized for expression in *L. paracasei*. The synthetic genes flanked by an upstream *BamH*I and a downstream *Sac*I restriction site for subsequent cloning.

In paper III, synthetic genes coding for human IL-21, hybrid IL-21/4 and CD40L were synthesized. The sequence of IL-21 and CD40L was codon optimized according to the codon usage of *L. paracasei.* The synthetic IL-21 and IL-21/4 genes were flanked by an upstream *BamH*I and a downstream *Sac*I restriction site for subsequent cloning.

In paper IV, the genes encoding the ARP3, VSV-G-tag, *prtP* and *apf* transcription terminator was synthesized. The ARP3 and *prtP* genes were codon optimized to reduce recombination between the two cassettes cloned in tandem. The synthetic genes were flanked by an upstream *EcoR*I and a downstream *Pvu*I restriction site while the VSV-G-tag was flanked by *Mlu*I and *Nhe*I for subsequent cloning.

In paper I, the GLP-1 peptide (98.3% purity, 31 amino acids) and its analogue GLP-1-Gly8 (97.5% purity, 31 amino acids) were produced synthetically by GenScript Corporation.

3.3 CLONING IN *LACTOBACILLUS*

Four shuttle expression plasmids were used for expression of therapeutic peptides. The expression vectors pAFβ100 and pAF100 mediate secretion, while the pAFβ900 and pAF900 mediate surface anchoring of the peptide. The detailed description of pAF and pAFβ plasmid series construction can be found in our previous work [30] and paper II. In most cases,

synthesized genes were digested with specific pairs of restriction enzymes and ligated into the expression vectors excised using the same restriction enzymes. Meanwhile, assembly PCR was also employed to facilitate changes of different parts of the expression cassettes (promoters, signal peptides or affinity tags). Details of the construction process can be found in the respective paper.

The expression plasmids were first transformed into *E. coli* DH5α by electroporation and the expression cassettes were verified by Sanger sequencing (Eurofins Genomics, Germany). The plasmids were subsequently transformed into *L. paracasei* BL23 [37, 184] by electroporation as previously described [37].

3.4 EXPRESSION ANALYSIS

3.4.1 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to quantify the expression level of IL-22 from lactobacilli and the secretion level of IL-10 from Colo205 cell line in paper II.

Flat-bottom 96 well plates (EIA/RIA, Costar) were coated with 1 μg/ml goat anti-mouse IL-22 antibody (R & D Systems) or goat anti-human IL-10 antibody (Sigma) overnight at 4°C, and then followed by blocking with 1% BSA in PBST (PBS containing 0.05% Tween 20) for two hours at room temperature. Serial dilutions of the *Lactobacillus* supernatant (1/10 to 1/1280) from Lp pAFβ100-IL22 or Colo 205 cell culture supernatant were added and incubated at room temperature for 2 h. Recombinant mouse IL-22 (R & D Systems) or human IL-10 (BD Pharmingen) was used to create a standard curve at concentrations of 500 ng/ml to 244 pg/ml. Plates were washed with PBST and a biotinylated goat anti-mouse IL-22 antibody (0.2 μg/ml, R & D Systems) or biotinylated goat anti-human IL-10 antibody (1 μg/ml, R & D Systems) was added and incubated at room temperature for 1 h. Plates were subsequently washed with PBST and incubated for one hour with streptavidin-alkaline phosphatase (1/1000 dilution, BD Pharmingen) at room temperature for detection. Diethanolamine buffer (1 M, pH=10.0) containing 1 mg/ml of pNPP (Sigma-Aldrich) was added to the wells. After 20 min incubation, absorbance was read at 405 nm in a Varioskan Flash microplate reader (Thermo Electron Corporation).

ELISA was also used to evaluate the binding of antibody fragments to rotavirus in paper III. The protocol was slightly modified as follows: ELISA plates were coated with anti-rotavirus HBC antibodies (5 μg/ml) for 2 h, prior coating with rotaviruses overnight at 4°C. ARP1 was detected using a mouse anti-E-tag antibody (1/2000) followed by an alkaline phosphatase conjugated rabbit anti-mouse antibody (Dako, 1/1000), and ARP3 was detected using a biotinylated anti-V5-tag antibody followed by alkaline phosphatase conjugated streptavidin (Becton Dickinson, 1/1000).

3.4.2 Western Blot

Western blot was used to detect the expression of peptides and cytokines from *Lactobacillus*. The antibodies used in this thesis are listed in Table 1.

Table 1 Antibodies used in the Western blot.

	Primary antibody	Secondary antibody	Target
Paper I	Mouse monoclonal anti-Glucagon-like peptide-1 (Mid-molecule specific, BioPorto), 0.5 μg/ml	HRP conjugated goat anti-mouse antibody (Dako A/S, Glostrup, Denmark), 1/1000	GLP-1
Paper II	Biotinylated goat anti-mouse IL-22 antibody (R & D Systems), 0.2 μg/ml	HRP conjugated streptavidin (BD Pharmingen, USA), 1/2000	IL-22
Paper III	Mouse monoclonal anti-E-tag antibody (Phadia AB, Uppsala, Sweden), 1 μg/ml	HRP conjugated goat anti-mouse antibody (Dako A/S, Glostrup, Denmark), 1/1000	E-tag (ARP1)
	Biotinylated mouse monoclonal anti-V5-tag antibody (AbD Serotec, Kidlington, UK), 1/5000	HRP conjugated streptavidin (BD Pharmingen, USA), 1/2000	V5-tag (ARP3)
Paper IV	Biotinylated rabbit anti-mouse IL-21 antibody (R & D Systems), 0.2 μg/ml	HRP conjugated streptavidin (BD Pharmingen, USA), 1/2000	IL-21
	Mouse monoclonal anti-E-tag antibody (Phadia, AB, Uppsala, Sweden), 1 μg/ml	HRP conjugated goat anti-mouse antibody (Dako A/S, Glostrup, Denmark), 1/1000	E-tag (CD40L)

The bacterial cultures were centrifuged when OD_{600} reached 1.0. The supernatant was filter-sterilized using a 0.2 μm filter and the pH was adjusted to 7.0. Then, 100 μl of supernatant was mixed with an equal volume of 2×Laemmli buffer (Bio-Rad) and boiled for 5 min. The bacterial pellets from 1 ml culture were washed twice with PBS followed by resuspension in 100 μl 2×Laemmli sample buffer and boiled for 10 min. The boiled samples were centrifuged at 16100×g to remove cell debris and the supernatant containing the soluble proteins was retained as a cell extract sample. Ten ng of the commercial recombinant peptide (positive control) and 20 μl of the supernatant or the cell extract were run on a 12% SDS-PAGE gel. The proteins were then transferred onto a nitrocellulose membrane (Hybond-ECL, GE Healthcare, UK). After overnight blocking with 5% skim milk in PBST, the membrane was incubated with primary antibody for 2 h, followed by washing 4 times with PBST, and then incubated with secondary antibody for 1 h. The signal was detected by chemiluminescence using the ECL Western blotting detection system (GE Healthcare).

Furthermore, the amount of IL-22 produced in the supernatant of *Lactobacillus* (Lp pAFβ100-IL22) cultures was quantified by Western blot densitometry and compared to commercial recombinant IL-22 protein (Paper II). In paper III, the amount of ARP1 and ARP3 fragments produced in the supernatant and cell extract of *Lactobacillus* cultures was also quantified by Western blot densitometry as compared to known concentration of affinity column purified ARP1 and ARP3 proteins.

3.4.3 Flow cytometry

Cell wall display of peptides and cytokines on the surface of *Lactobacillus* was confirmed by flow cytometric analysis.

Table 2 Antibodies used in the flow cytometry.

	Primary antibody	Secondary antibody	Target
Paper I	Mouse monoclonal anti-Glucagon-like peptide-1 (Mid-molecule specific, BioPorto), 1/200	FITC-conjugated goat anti-mouse antibody (Jackson Immunoresearch Lab., West Grove, USA), 1/200	GLP-1
Paper II	Biotinylated goat anti-mouse IL-22 antibody (R & D Systems), 1 µg/ml	FITC-conjugated goat anti-mouse antibody (Jackson Immunoresearch Lab., West Grove, USA), 1/200	IL-22
Paper III	Mouse monoclonal anti-E-tag antibody (Phadia AB, Uppsala, Sweden), 1/100	FITC-conjugated goat anti-mouse antibody (Jackson Immunoresearch Lab., West Grove, USA), 1/200	E-tag (ARP1)
	Biotinylated mouse monoclonal anti-V5-tag antibody (AbD Serotec, Kidlington, UK), 1/400	FITC conjugated streptavidin (Biolegend, San Diego, CA), 1/200	V5-tag (ARP3)
Paper IV	Mouse monoclonal anti-E-tag antibody (Phadia, AB, Uppsala, Sweden), 1 µg/ml	FITC-conjugated goat anti-mouse antibody (Jackson Immunoresearch Lab., West Grove, USA), 1/200	E-tag (CD40L)

Fifty µl of *Lactobacillus* cultures grown to an OD_{600} of 1.0 were pelleted by centrifugation and washed twice in PBS. Bacteria were incubated with a primary antibody for 30 min on ice. After two PBS washes, the cells were incubated with a secondary antibody (or streptavidin) on ice for 30 min in the dark. The primary and secondary antibodies were diluted in PBS containing 1% BSA. After washing three times with PBS, samples were resuspended and

fixed in 400 µl 2% paraformaldehyde and analyzed using a FACS Calibur machine (Becton Dickinson, Franklin Lakes, NJ). All results were further analyzed by the FlowJo™ software.

3.5 PLASMID LOSS ASSAY (PAPER II AND IV)

To evaluate whether the non-stained population observed in flow cytometry of the anchored cytokine expressing lactobacilli is due to the plasmid loss, an assay was carried out to test the plasmid loss of cytokine expressing lactobacilli during growth under antibiotic pressure. The bacteria were cultured under normal conditions with antibiotic supplemented to an OD_{600} =1.0, followed by dilution of 10^6 fold in MRS medium, and then plated on MRS plates with or without specific antibiotic. After two days growth anaerobically, the colonies on each plates were counted. The experiment was performed in duplicate.

3.6 PROTEIN PURIFICATION

In paper I, wt 5×GLP-1 and trp 5×GLP-1 were expressed in *E. coli* with His-tag and purified to approximately 85% purity by GenScript (Piscataway, NJ). The *Lactobacillus*-produced Lp wt 5×GLP-1 and Lp trp 5×GLP-1 were purified from the culture supernatant of lactobacilli with anti-E-tag monoclonal antibodies coupled to an NHS-HiTrap sepharose column (GE-healthcare) according to the manufacturer's instructions. The eluate was concentrated to a volume of 0.5 ml using an Amicon Ultra-4 3K centrifugal filter (Millipore, Billerica, USA). The purity of purified 5×GLP-1 was verified on SDS-PAGE and the concentration was determined by the Micro BCA Protein Assay Kit (Pierce, Rockford, USA) as compared to known concentration of *E. coli* purified 5×GLP-1.

In paper III, the ARP1 and ARP3 were also purified from supernatant of *Lactobacillus* pAF1200 using NHS-HiTrap sepharose column (GE-healthcare) coupled to anti-E-tag or anti-V5 monoclonal antibodies according to the manufacturer's instructions.

3.7 *IN VITRO* MODEL

3.7.1 Insulinotropic activity on HIT-T15 cells (paper I)

The hamster β-cell line HIT-T15 (ATCC CRL-1777) is a popular *in vitro* model to study insulinotropic drugs [185]. HIT-T15 cells (passages 81–82) were maintained in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% Penicillin/Streptomycin (Invitrogen AB). The cells were subcultured in 6-well plates at a density of 1×10^5 cells/well, and then cultured for 5 days before use in the experiment.

The cells were incubated twice with 2 ml Krebs-Ringer Buffer (KRB) containing 0.1% BSA at 37°C for 30 min, rinsed once with 2 ml KRB containing 0.1% BSA and incubated with 1 ml KRB buffer (either without glucose or with 5.6 mM glucose) containing digested pentameric GLP-1 purified from *Lactobacillus* or *E. coli* for 60 min at 37°C. 100 nM of synthesized GLP-1-Gly8 peptide was used as a positive control. Following incubation, the cell-free supernatants were subjected to insulin quantification using a rat insulin ELISA kit (Mercodia AB).

3.7.2 IL-22 biological activity assay (paper II)

The bioactivity of *Lactobacillus* produced mouse IL-22 was measured by its ability to stimulate IL-10 secretion in the human colon cancer cell line Colo 205 [186]. Colo 205 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin. Cells grew loosely attached or in suspension in flasks till confluency, they were then seeded in 6-well plates at a density of 1×10^6 cells/well.

Two strains of *Lactobacillus*, Lp pAFβ100-IL22 secreting IL-22 and Lp pAFβ900-IL22 producing surface anchored IL-22, were tested. The wild type *L. paracasei* BL23 strain was used as a negative control. The supernatants of Lp pAFβ100-IL22 and wild type *Lactobacillus* from 10 ml MRS culture were harvested at OD_{600} =0.8 and adjusted to pH=7.0 before use. The bacterial pellets of Lp pAFβ900-IL22 and wild type *Lactobacillus* were washed twice with PBS, resuspended in PBS and treated with UV light using a cross linker (for 2 min at 120 millijoules/cm^2) to kill the bacteria before use. Recombinant mouse IL-22 (R & D Systems), dilutions of bacterial culture supernatant or killed bacteria was added to Colo 205 cells in a total volume of 2 ml. The cells were incubated at 37°C for 22 hours, and then the supernatant was collected and tested for human IL-10 concentrations by ELISA.

3.7.3 PBMCs stimulation and enzyme-linked immunospot (ELISPOT) assay (paper III)

3.7.3.1 PBMCs separation

Heparinized peripheral venous blood was obtained from healthy blood donors and IgAD patients. PBMCs were isolated and cryopreserved as described by Kreher *et al*. [187]. Thawed PBMCs were washed twice with IMDM (Gibco) and cultured in IMDM with GlutaMAX™ supplement, 1% penicillin-streptomycin, and 10% heat-inactivated fetal bovine serum (Gibco) at 37°C in the presence of 5% CO_2.

3.7.3.2 PBMCs stimulation

To measure the biological activity of *Lactobacillus* produced IL-21, IL-21/4 and CD40L, the strains were grown in MRS to an OD_{600}=1.0 and centrifuged for 10 min. The bacterial pellets were washed twice in PBS while the culture supernatant was filter-sterilized and pH adjusted to 7.0. The supernatant was concentrated 10 times using Amicon Ultra-4 10K centrifugal filter (Millipore). PBMCs (3×10^6) were co-cultured for 6 days with a mixture of either: 1) concentrated culture supernatant of *Lactobacillus* secreting IL-21 or IL-21/4 with 20 ng/mL MegaCD40L (Enzo Life Sciences) or 2) bacteria from Lp pAF900-CD40L with 10 ng/µl human IL-21 (R & D Systems). The supernatant or pellet of the non-expressor *L. paracasei* BL23 was used as negative controls. The positive control consist of MegaCD40L (20 ng/mL) and recombinant IL-21 (50 ng/ml) only.

3.7.3.3 Enzyme-linked immunospot (ELISPOT) assay

MultiScreen$_{HTS}$ filter plates (EMD Millipore) were prewet with 20% ethanol, rinsed 3 times with sterile PBS, and coated with either polyclonal rabbit anti–human IgA or IgG antibody (10 μg/mL, SouthernBiotech) overnight at 4°C. After washing, plates were blocked with IMDM containing 10% fetal bovine serum and 1% bovine serum albumin (BSA, Sigma-Aldrich) for 2 h. On day 6 of stimulation, PBMCs were washed once with culture medium and plated at 1×10^5 cells/well in ELISPOT plates. The plates were incubated for 48 h at 37°C in the presence of 5% CO_2. Thereafter, the plates were washed 6 times by PBS containing 0.01% Tween20 (PBST). The detection antibodies, goat anti–human IgG-alkaline phosphatase and goat anti–human IgA-alkaline phosphatase (SouthernBiotech), were diluted in PBS containing 0.5% BSA at 2 μg/ml, and then 60 μl were added to each well. After incubation overnight at 4°C, the plates were washed 6 times with PBST. Spot development was carried out using the BCIP/NBT Liquid Substrate System (Sigma-Aldrich) and then analyzed on the AID EliSpot Reader using AID reader software (AID GmbH, Germany).

3.8 ANIMAL MODEL (PAPER I)

Goto-Kakizaki (GK) rats, which is a spontaneous non-obese type 2 diabetes model [188], were used for testing the activity of synthesized 5×GLP-1 peptide and *Lactobacillus or E. coli* produced 5×GLP-1. GK rats were housed at 22°C with an alternating 12-hour light-dark cycle and access to standard pellet diet (B&K Universal) and water.

For testing the activity of monomeric synthetic GLP-1-Gly8 peptide, GLP-1-Gly8 was given subcutaneously to GK rats at a dose of 0.5–1.5 mg/kg body weight. For testing the activity of *E. coli* produced 5×GLP-1 peptide, rats were cannulated with a catheter inserted into the small intestine to avoid the peptide being digested before reaching the intestine. For testing the *Lactobacillus* producing pentameric GLP-1, rats were gavaged with two doses of *Lactobacillus* strains (10^{10} cfu) daily.

3.8.1 Intraperitoneal glucose tolerance test (IPGTT)

An IPGTT was used to measure the clearance of glucose from the GK rat after administration of GLP-1 peptide or *Lactobacillus* expressing 5×GLP-1. GK rats were fasted overnight before the experiment. A solution of glucose (2 g/kg of body weight) was administered by intraperitoneal injection, followed by blood glucose measurement at different time-points during the subsequent 3 hours. Blood was obtained by the tail-prick method and measured using a glucometer.

3.8.2 Oral administration of lactobacilli

To test the anti-diabetic effect of 5×GLP-1 expressing *Lactobacillus*, each group of GK rats (n = 6) was given either PBS or *Lactobacillus* strains (KKA101, KKA394 or KKA403) by gavage continuously for seven days. The lactobacilli were grown to the logarithmic phase,

pelleted by centrifugation, washed twice with PBS and finally resuspended in PBS at 10^{10} cfu/ml. One ml of either PBS or *Lactobacillus* suspension was given to each rat.

Non-fasting blood glucose levels were measured by tail-prick method every morning. At the end of the experiment (the morning of day 7 after whole night fasting), rats were given the last gavage of lactobacilli and then subjected to an IPGTT with 2 g glucose/kg body weight 15 min later. The serum sample was taken from the tail vein at days 0, 6 and 13. The body weight of the rats was monitored every day during the 14-day experimental period.

3.9 STATISTICS

Two-way repeated ANOVA followed by the Student-Bonferroni multiple-range test was used to estimate the significance of differences for glycaemia between groups during IPGTT and the *Lactobacillus* treatment period. One-way ANOVA followed by the Student-Bonferroni multiple-range test was used to estimate the significance of differences between groups for area under the curve of blood glucose levels during IPGTT. Difference in insulinotropic effect between purified peptides from *E. coli* and *Lactobacillus* was analyzed by the unpaired t-test with Welch's correction. Differences were considered statistically significant if $p<0.05$. Data were analyzed using Excel (Microsoft Corp.) and GraphPad Prism v6.0 (GraphPad Software).

4 RESULTS AND DISCUSSION

4.1 PAPER I

This paper describes our first attempt to deliver a therapeutic peptide using our *Lactobacillus* expression system directly in the gastrointestinal tract. The incretin peptide GLP-1 was chosen due to some reasons: 1) GLP-1 is naturally released from the L-cells in the ileum and colon, while oral delivery of peptides followed by uptake through the intestine would more likely mimic physiological GLP-1 secretion than the current subcutaneous injection routine. 2) It has been shown that orally administered GLP-1 is rapidly absorbed and markedly affects the glucose level in healthy human subjects [76] when a large dose (2 mg) is given. 3) *Lactobacillus* strains can colonize the gastrointestinal tract temporarily, providing a continuous supply of GLP-1 peptide, which would facilitate absorption through epithelial cells.

4.1.1 From GLP-1 to 5×GLP-1

Based on a previous report [71] and the activity of the peptide on the isolated rats' islet and in subcutaneously injected GK rats, GLP-1-Gly8 was chosen to be expressed by *Lactobacillus.* Previous reports have also shown that the intact N-terminal histidine of the mature GLP-1 peptide is important for its insulinotropic effect [189, 190]. However, the N-terminus of *Lactobacillus* naturally secreted proteins rarely start with a histidine [25]. Since the protein secretion is strongly determined by the signal peptide, 8 different signal peptides from *Lactobacillus* naturally secreted proteins were selected for fusion to the GLP-1-Gly8 gene based on a prediction of cleavage sites by SignalP [25, 26]. However, none of them led to the secretion of GLP-1-Gly8 with a correct N-terminal cleavage. This failure led us to design a pentameric form of GLP-1 (5×GLP-1) which consists of five consecutive GLP-1-Gly8 monomers in tandem. Fourteen extra amino acids from the APF fusion partner were left on the N-terminal of 5×GLP-1 to facilitate a good cleavage and secretion of this peptide, and a tag was added to facilitate purification or detection. Most importantly, the 5×GLP-1 was expected to be digested by the intestinal trypsin into five active monomers of GLP-1 (starting with a histidine) when it is released into the intestine. A wildtype pentameric GLP-1 (wt 5×GLP-1) and a trypsin-stabilized version of the pentamer (trp 5×GLP-1, which includes two mutations in the GLP-1-Gly8 monomers to stabilize it against tryptic digestion within the monomer) were constructed. We believed that this design would guarantee the intact N-terminal of GLP-1-Gly8 delivered into the GI tract, while also increase the amount of functional peptide being delivered.

4.1.2 5×GLP-1 analogues are bioactive

The 5×GLP-1 analogues were both successfully expressed in a secreted form and anchored on the surface of lactobacilli. The trypsin digested 5×GLP-1 produced both by *Lactobacillus* and *E. coli* showed a glucose dependent insulinotropic effect on the pancreatic β-cell line.

This *in vitro* result proved that 5×GLP-1 expressed from *Lactobacillus* and *E. coli* have a similar bioactivity. This result was of importance for the following *in vivo* experiment, since we cannot purify a large amount of 5×GLP-1 from lactobacilli but the purification from *E. coli* is rather cheap and efficient. 5×GLP-1 was purified from *E. coli*, and delivered by intestinal intubation to GK rats, resulting in a significant improvement of glycemic control demonstrated by IPGTT.

4.1.3 Oral delivery of 5×GLP-1 by *Lactobacillus* to the diabetic rats

When the 5×GLP-1 expressing lactobacilli were given by oral gavage twice daily to GK rats for 7 days, only the rats receiving non-expressing lactobacilli (negative control) showed a significant decrease in blood glucose as compared to the PBS control. The anchored trp 5×GLP-1 showed a similar trend to lower the blood glucose level, but not to a statistically significant degree. Similar results were observed in a repeat of this experiment with an extended 14-days treatment.

The non-expressor *L. paracasei* BL23 could significantly lower the blood glucose level, which can be explained by the anti-diabetic effect of lactobacilli (either lower plasma glucose levels or improve insulin resistance in diabetic animal models) reported recently by many groups [16, 17, 82-84].

The limited amount of GLP-1 delivered by lactobacilli might be the main reason that no significant difference was observed compared to the non-expressor lactobacilli. The theoretical maximum amount of GLP-1 given by the anchored strain is 1.5 μg GLP-1 per rat [30], which is significantly lower than the amount of purified *E. coli* 5×GLP-1 (around 1 mg/rat) that was given through the intestinal catheter.

Secondly, some lactobacilli might lose their ability to express 5×GLP-1 when delivered in the intestine since the antibiotic pressure which maintains the plasmid is absent *in vivo*. The plasmid loss rate was approximately 40% for the anchored trp 5×GLP-1 strains and 65% for the secreted 5×GLP-1 strains when passing through the GI tract of the GK rats. This problem may be solved by integrating the 5×GLP-1 gene into the *Lactobacillus* chromosome and thereby stabilizing its expression.

Thirdly, it is worth noting that the secreted and surface anchored strains behave differently in the lactobacilli feeding experiment as only the trp 5×GLP-1-anchored lactobacilli (and non-expressor lactobacilli) demonstrated a trend towards daily blood glucose level reduction. In our previous study involving delivery of antibody fragments, both secreted and surface anchored constructs were used, but the majority of study showed that the anchored display is more effective in reducing infection than the secreted one *in vivo* [37-40, 191]. In this study, the anchored lactobacilli act like beads already loaded with 5×GLP-1 peptides that can be immediately proteolytically released upon contact with trypsin in the intestine, while on the contrary, the secreted lactobacilli depend on the secretion of the peptide *in situ* in the intestinal tract. Since the non-expressor lactobacilli is more effective in reducing daily glucose level, there might be an undefined mechanism whereby surface molecules involved

in the anti-diabetic effect of lactobacilli had been hindered in the secreting strain, but not in the surface anchored strain. However, we would normally expect that surface anchoring influences more the surface structures than the secreted construct. This interesting observation might lead to future studies investigating the anti-diabetic effect of *Lactobacillus* strains.

Finally, we did consider the expression level from the beginning by designing a pentameric GLP-1 to increase the amount of peptide that each *Lactobacillus* could deliver. We also expected a higher local concentration of 5×GLP-1 in the intestine when delivered by *Lactobacillus*. Even so, further work is still needed to increase the expression level of GLP-1 by lactobacilli in order to see a significant insulinotropic effect *in vivo*.

4.2 PAPER II

IL-22 plays a prominent role in epithelial regeneration and dampening of chronic inflammatory responses by protecting intestinal stem cells from immune-mediated tissue damage. IL-22 is referred to as a cytokine that has a therapeutic potential in GVHD [192], which is a frequent complication following allogeneic stem cell transplantation. Here we chose to express mouse IL-22 in lactobacilli both as a secreted product and covalently anchored on the surface of lactobacilli. If the bioactivity of lactobacilli produced IL-22 can be proved, later it could be used for delivery of IL-22 directly to the intestinal mucosa as a new therapeutic strategy for GVHD.

4.2.1 Expression and display of IL-22

To express the mouse cytokine IL-22, a new expression vector pAFβ, which is very similar to the pAF series vector, was constructed. The pAFβ series, based on the pIAβ8 shuttle plasmid, has a different antibiotic marker for selection (chloramphenicol instead of erythromycin). Furthermore, the expressed IL-22 has only 6 extra-amino acids on the N-terminus following signal peptide cleavage compared to 13 extra-amino acids if the pAF series would be used. We were not sure if this change would lead to a better expression and secretion, but it would make the expressed IL-22 more similar to the original peptide, therefore maybe resulting in a better bioactivity.

Successful expression of mouse IL-22 was demonstrated by Western blot and flow cytometry. When grown in MRS medium, *Lactobacillus* acidifies the growth medium from 6.3 to pH 4.5. Since IL-22 is a member of the IL-10 family of cytokines and IL-10 is highly sensitive to low pH less than 5.5 [193], we tested the sensitivity of IL-22 to low pH caused by lactic acid produced by *Lactobacillus*. Slight increase (approximately 0.3-fold) in IL-22 concentration was observed when the lactobacilli expressing IL-22 were grown in pH adjusted medium. However, these results are not significant enough to determine if the slightly reduced IL-22 level in pH non-adjusted MRS is due to the sensitivity of IL-22 to low pH. It is however certain that the effect of pH on IL-22 production is not as important as for IL-10.

The display of mouse IL-22 on the surface of *Lactobacillus* strain was demonstrated by flow cytometry but only in a small proportion (20%) of the lactobacilli. A non-displayed population has been previously observed using flow cytometry in our anchored 5×GLP-1 expressing lactobacilli (<24%) but not to such a high level (80%). A large population of non-stained lactobacilli was more recently observed when we expressed cytokines (see paper IV). It is worth noting that these strains were also growing very slow in the liquid MRS medium with antibiotics. The plasmid loss assay revealed that 60% of the secreted IL-22 strain and 75% of the anchored IL-22 strain lost their plasmid during growth even under antibiotic pressure, which is corresponding to the flow cytometric results of IL-22 surface display. We may speculate that the slow growth of these strains could be due to survival pressure caused by expressing the cytokine which lead to plasmid loss.

4.2.2 Bioactivity of IL-22

The *Lactobacillus* secreted and anchored mouse IL-22 were both proven to be biologically active, as determined by their ability to induce IL-10 secretion in an *in vitro* model (Colo 205 human colon cancer cell line). Our results suggest that IL-22 expressing lactobacilli may potentially be a useful mucosal therapeutic agent for the treatment of GVHD, provided that higher expression levels can be achieved.

The dose of IL-22 that reduces intestinal pathology and mortality from graft-versus-host disease was shown to be 1.5×10^{14} molecules when using intraperitoneale injection. To be able to test our *Lactobacillus* strains in the GVHD mice model in future, the current amount of IL-22 peptide that lactobacilli could delivered is probably still not sufficient (maximum 2.2×10^{13} molecules) in order to reach the Intestinal stem cell compartment at the bottom of the crypt of the intestinal epithelium. Furthermore, plasmid loss was observed during growth in the culture medium and it is expected that such plasmid loss will increase in the absence of antibiotic selective pressure *in vivo*. Further experiments should be performed to improve the IL-22 delivery by integrating the expression cassette into the *Lactobacillus* chromosome and thereby stabilizing its expression.

4.3 PAPER III

Anti-rotavirus specific VHH fragments, denoted as ARP1 and ARP3, derived from a rhesus rotavirus immunized library from llamas, were previously expressed as monomers as well as homo- and hetero- dimers on the cell surface of lactobacilli [39, 40]. In this paper, we developed co-expression vectors containing two expression cassettes in tandem for expression of two different VHH antibody fragments against rotavirus in *L. paracasei* BL23.

4.3.1 Development of co-expression vectors

Engineered *Lactobacillus* producing either surface-anchored ARP1 or ARP3 were previously constructed and shown to be therapeutically effective in the rotavirus infection mouse model. There are limitations for one VHH fragment targeting a single epitope due to reduced cross-reactivity to circulating viral serotypes and potentially viral escape mutants. Therefore, we

believed that targeting multiple epitopes might improve the efficacy. To generate *Lactobacillus* expressing two rotavirus-specific VHH fragments (ARP1 and ARP3) in secreted and surface anchored forms, could certainly increase the binding to rotavirus and aggregation (due to binding to distinct epitopes). Additionally, if the virus acquires mutation on one binding site, the other VHH would retain its binding ability to the virus. Three distinct double expression cassettes were designed where the ARP1 and ARP3 were produced both secreted (pAF1200) in the medium, both covalently anchored (pAF1400) on the cell surface, or one secreted and the other anchored (pAF1300). To distinguish the co-produced ARP1 and ARP3 by *Lactobacillus*, different tags were selected and fused to each of them.

4.3.2 Binding activity of co-expressed VHHs

The expression and surface display of anchored ARP1 and ARP3 fragments on *L. paracasei* BL23 cells was verified by Western blot and flow cytometry. The level of expression as determined by Western blot densitometry was similar between co-expressor lactobacilli and lactobacilli producing one VHH fragment. Both VHH fragments were shown to be displayed in equal amounts on the surface of lactobacilli producing one or two fragments by flow cytometric analysis.

Furthermore, anchored ARP1 and ARP3 produced by lactobacilli were shown to bind to all the tested strains of human and simian rotavirus in flow cytometry. Culture supernatant from lactobacilli producing secreted ARP1 and ARP3 were also broadly cross-reactive against human and simian rotavirus strains in ELISA.

As a pediatric pathogen, rotavirus cause severe diarrhea in infants and is associated with a high mortality rate. Co-expression of two distinct VHH antibody fragment in *Lactobacillus* could increase the chance of neutralization through binding to different epitopes of the virus, meanwhile decrease the cost of production since there is no need of growing distinct batches of single-expressor bacterial cultures. Due to the broad cross-reactivity with many G/P combinations and the reduced production cost, lactobacilli co-expressing antibody fragments represent an alternative therapy for the developing world where people are infected with various genotypes.

Most importantly, these co-expression platforms were designed with different restriction sites flanking the VHH genes, which will facilitate the cloning and delivery of various other therapeutic peptides by lactobacilli in the future. In paper IV, this co-expression vector was used to express IL-21 and CD40L in the same bacteria.

4.4 PAPER IV

Our previous studies proved that IL-21/CD40L stimulation may be an effective therapy for IgA deficient patient [140, 154]. In this paper, we are evaluating the possibility of developing an IL-21/CD40L-based *Lactobacillus* delivery system to induce immunoglobulin production in the gastrointestinal tract from patients with IgAD. As a first step, we expressed IL-21(or

hybrid IL-21/4) and CD40L individually, or co-expressed IL-21 and CD40L in *Lactoacillus paracasei* BL23, and tested if they could induce IgA secretion in PBMCs.

4.4.1 *Lactobacillus* constructs and expression of IL-21 (IL-21/4) and CD40L

A hybrid IL-21/4 was designed to combine the receptor-binding ability of IL-21 and IL-4 based on our previous studies which showed that IL-4 can potentiate the immunoglobulin-production induced by IL-21 [140, 154]. The chimeric molecule (hybrid IL-21/4 protein), together with IL-21, were first cloned into the pAFβ100 vectors due to the same reason that we discussed in paper II (fewer extra amino acids on N-terminus after secretion). However, the expression level was not as good as we expected. Human IL-21 has not yet been expressed by prokaryotes except in *E.coli* as inclusion body protein. We put much effort into trying to express IL-21 in lactobacilli by using different signal peptides, promoters (inducible expression promoter), fusion to a tag or other protein (human serum albumin domain III)(Table 3). However, all the attempts failed except when we cloned IL-21 into our pAF100 vector. This could possibly be attributed to the additional seven amino acids left at the N-terminus of the cytokine following cleavage of the signal peptide in pAF100 or plasmid stability. However, a strong signal was also observed in the cell pellet extract, indicating that the secretion of IL-21 from lactobacilli could be further optimized.

Table 3 Different strategies employed to try to express IL-21(IL-21/4)

	Constructs	Expression	Secretion
IL-21	IL-21 secreted (extra N-ter aa)	Yes, weak smear	Yes, weak smear
	IL-21 anchored (extra N-ter aa)	Yes, weak band	Yes, weak band
	IL-21 his-tag (C)	No	No
	IL-21 his-tag (N)	No	No
	IL-21 secreted (direct fusion)	No	No
	IL-21 secreted (direct fusion his-tag C)	No	No
	IL-21 streptag N-terminal	No	No
	IL-21 secreted Usp45 P+SP [a]	No	No
	IL-21 secreted Sakacin P [b]	Yes	No
	IL-21 N-ter HSA-D3 fusion	Yes, weak band	Yes, weak band
	IL-21 C-ter HSA-D3 fusion	Yes, weak band	Yes, weak band
IL-21/4	IL-21/4 secreted (extra N-ter aa)	Yes, weak smear	Yes, weak smear
	IL-21/4 anchored (extra N-ter aa)	Yes, weak band	Yes, weak band
	IL-21/4 streptag N-terminal	No	No
	IL21/4 E-tag secreted	No	No

All the constructs were made in pAFβ100 or pAFβ900 vector which based on pIAβ8 plasmid. Except [a] in pAF100 and [b] in pVPL3017 [194]. P: promoter; SP: signal peptide. HSA-D3: human serum albumin domain III.

Human CD40L (with an E-tag fused at the C-terminal) was expressed anchored on the surface of *Lactobacillus* using the pAF900 vector, and surface display was confirmed by flow cytometric analysis. It is worth mentioning that 45% of the bacterial cells were not stained with the anti-E-tag antibody while only 14% were not stained for the positive control *Lactobacillus* expressing ARP1. We showed that approximately 57% of the bacteria lost their plasmid during growth in liquid, antibiotic supplemented MRS, which corresponds to the flow cytometric results. It indicates that the low IL-21 expression level could also be due to plasmid loss.

The co-expression cassette developed in paper III was used to simultaneously produce both secreted IL-21 and anchored CD40L on the surface of lactobacilli. The aim of the co-expression is to have both IL-21 and CD40L delivered at the same time to naïve B cells in the intestine. This moment could happened in the intestine when the intestine-resident dendritic cells sample bacteria in the lumen through transepithelial dendrites, or the live bacteria are transported across the epithelial barrier through M cells in the Peyer's patches [195].

The co-expression cassette for IL-21/CD40L (pAF1300) is a fusion of pAF100 and pAF900 cassettes in tandem, which have exactly the same promoter and terminator. However, the attempt of constructing lactobacilli co-expressing IL-21/4 and CD40L was not successful and resulted in loss of either IL-21/4 or CD40L gene expression probably due to plasmid rearrangement. Although such plasmids were previously shown to successfully express two VHH antibody fragments in paper III, rearrangement of the vector between the homologous sequences could possibly increase when the *Lactobacillus* strain is facing the pressure on growth caused by expressing human cytokines. The CD40L gene could be fused with another promoter and terminator to prevent rearrangement between homologous regions in the co-expression vector.

4.4.2 Bioactivity test of IL-21 (IL-21/4) and CD40L

The lactobacilli expressing IL-21 (IL-21/4) and CD40L were tested for their ability to induce IgA switching in PBMCs from a healthy donor or IgAD patients' by using a single cell ELISPOT assay. The 10-fold concentrated supernatant of *L. paracasei* expressing IL-21/4 using pAFβ100 stimulated both IgG and IgA production in a healthy donor and IgA production in two IgAD patients' PBMCs. The concentrated supernatant of *L. paracasei* expressing IL-21 using pAFβ100 failed to stimulate IgG or IgA production in PBMCs probably due to a low expression level of IL-21 in the supernatant even after concentration. However, the concentrated supernatant of *L. paracasei* expressing IL-21 using pAF100, with higher level of IL-21, could stimulate both IgG and IgA production in healthy donor PBMCs. The *Lactobacillus* producing anchored CD40L along with the commercial recombinant human IL-21 was also shown to induce both IgG and IgA production in a healthy blood donor's PBMCs.

Our results thus showed that *Lactobacillus* produced IL-21, IL-21/4 and anchored CD40L are bioactive. This could eventually lead to a therapy where orally administered lactobacilli

producing both surface anchored CD40L and secreted IL-21 would induce IgA switching in B cells from IgAD patients and ultimately induce secretion of IgA in the mucosal tissues. Intestinal biopsies from IgA deficient patients will be prepared in future, both as whole tissue and single cell suspensions, stimulated with *Lactobacillus* delivering CD40L and IL-21 (or IL-21/4) to investigate cytokine mediated IgA production.

5 CONCLUSIONS

The aim of this thesis is to explore the possibility of using lactobacilli as vectors for delivery of peptides in the gastrointestinal tract for therapy against type 2 diabetes and other immune-related diseases.

In **paper I**, a trypsin cleavable oligomers–pentameric GLP-1 was successfully expressed both in a secreted form and anchored on the surface of *L. paracasei* BL23. The pentameric GLP-1 proved to be bioactive both *in vitro* and in the intestine of diabetic rat following digestion by intestinal trypsin. When the lactobacilli were given by gavage to the diabetic rats, the non-expressor *L. paracasei* BL23 showed a significant anti-diabetic effect but GLP-1 expression did not provide an additional insulinotropic effect.

In **Paper II**, the secreted and surface anchored mouse IL-22 was expressed by *L. paracasei* BL23. The biological activity of IL-22 produced by *Lactobacillus* was demonstrated *in vitro.*

In **paper III**, a co-expression vector to produce two rotavirus-specific VHH antibody fragments in *Lactobacillus* was constructed. VHHs were expressed in secreted and surface anchored forms and their ability to bind to various rotavirus serotypes was demonstrated.

In **paper IV**, IL-21 (or hybrid IL-21/4) and CD40L were expressed individually, or co-expressed in *L. paracasei* BL23. We showed that *Lactobacillus* expressing IL-21, IL-21/4 and CD40L individually are bioactive in inducing IgA secretion in PBMCs from healthy donors.

6 FUTURE PERSPECTIVES

The present thesis advanced the knowledge on the use of *Lactobacillus* for delivery of therapeutic peptides in the GI tract with a focus on the metabolic disorder (type 2 diabetes) and immune-related diseases (GVHD, IgAD).

To successfully deliver the peptide drugs in the GI tract, at least three main issues need to be considered. First, the stability of the peptide, since most of the peptides are susceptible to gastrointestinal proteases. We expected that *Lactobacillus in situ* delivery could reduce the peptide exposure to gastric acid, bile and digestive enzymes [196, 197]. Moreover, in paper I, we manipulated the 5×GLP-1 sequence to utilize the intestinal trypsin for releasing bioactive GLP-1 peptides. This strategy could be applied for *Lactobacillus* delivery of other peptides with a simple structure in the future. For the cytokines (paper II and IV) with more complex structure, bioactive cytokines were successfully expressed by lactobacilli without any amino acid modifications. Further optimization of the amino acid sequence could be performed in order to increase their stability in the GI tract provided that more knowledge on the cytokine structure is available.

Secondly, the peptide absorption in the GI tract is generally low due to their high molecular weight and low lipophilicity [198]. Low bioavailability of many orally delivered peptides could be partly attributed to the low permeability of the intestinal barrier [199]. Various absorption enhancers and cell penetrating peptides have been investigated before [200], and we also tried to fuse a 16 amino acid hydrophobic penetratin domain [201] at the C-terminus of GLP-1 (paper I, unpublished data). However, a lower bioactivity of the peptide was observed *in vitro* compared to the original GLP-1, indicating that the efficacy of the therapeutic peptide might be affected by the fused hydrophobic peptide. Future studies of oral peptide delivery should still focus on enhancing the absorption from the GI tract.

Lastly but most importantly, the amount of peptides that we can deliver to the gut is probably not enough due to the low expression level of our *Lactobacillus* delivery system. As in paper I, *Lactobacillus* gavage can deliver a maximum of 1.5 μg GLP-1 per rat, but the effective dose is at the mg level. The attempt of delivery of therapeutic peptides (GLP-1) was not as successful as delivery of antibody fragment (VHH) against intestinal infections [39-41] *in vivo*, which could be due to the following reasons: 1) for neutralization of pathogens, antibody fragments are not required at such a high level; 2) the antibody neutralization takes place in the gut lumen, but the therapeutic peptides need to cross the epithelial barrier to reach their receptors. Therefore, the expression system in lactobacilli should be improved in the future in order to increase their capability to deliver large amount of peptides.

Our current expression system in lactobacilli has a constitutive expression promoter which controls the expression of *apf* gene. This promoter should be strong since APF is one of the most abundant secreted proteins from *Lactobacillus crispatus* [202]. Therefore, it might not be that promising to explore additional promoters which could drive higher expression level. Low expression could also be due to that the expressed protein is an extra metabolic burden

for the bacteria. In the future, we might need an inducible promoter in our *Lactobacillus* delivery system, to reach a high expression level. The two currently best characterized inducible expression systems in lactobacilli, the NICE system [203] and the sakacin-based expression system [21] are not tightly controlled in many *Lactobacillus* strains, resulting in leakage which lead to a final low expression level. A better choice could be an inducible expression system based on riboswitches, which is a regulatory domain typically found in the 5´UTR region of mRNA, controlling the downstream gene expression at the translational level by binding small metabolites [204, 205]. Synthetic riboswitches could be designed to bind small metabolites (vitamin B12, thiamin pyrophosphate, nicotinamide etc.) in the GI tract and turn on the expression of peptides from engineered lactobacilli.

The selection of a strain that closely adheres to the intestinal epithelium and persist for a longer period might also increase the efficacy of the *Lactobacillus* delivery system for therapeutic peptides. Although the orally administrated probiotic lactobacilli only transiently colonize the intestinal tract [206], some strains like *L. rhamnosus* GG can persist for prolonged periods (up to two weeks depending of the individual) than others [18, 206-208] and therefore, should be a more appealing strain to deliver peptides. In addition, therapeutic peptides could be expressed by an indigenous *Lactobacillus* strain isolated from the animals since the strain should establish itself better in the gut when evaluating the therapeutic effect in animal model [209]. The selection of the *Lactobacillus* strains is however highly dependent on their transformability and the protocol of transformation might need to be established for each strain.

Plasmid loss was observed during the growth of lactobacilli producing cytokines (paper II and IV), and it might be one of the reasons leading to a lower expression level of these cytokines. Considering that the plasmid loss rate will be even higher when delivered in the GI tract, chromosomal integration of the expression cassette is preferable before *in vivo* testing in animal models and is a prerequisite for therapeutic applications in human. We tried to integrate the IL-22 gene on the chromosome using site specific chromosomal integration method as previously developed [30]. This method is however time consuming and we were not successful. We are also trying other methods for genomic integration of cytokine genes, like using a replicable plasmid to carry out homologous recombination, and then using another plasmid carrying the CRISPR/Cas9 system [194] to select the bacteria with an inserted gene. One problem could occur during the attempt of chromosomal integration: Since lactobacilli prefer to stop dividing in the medium with antibiotic rather than keeping the cytokine expressing plasmid, this probably means the cytokine expression causes a very high stress for the bacteria and therefore, the chromosomal integration is likely to fail too. Even if integration is achieved, the strain might try to avoid expressing the cytokine through other methods like point mutations or genomic rearrangements that lead to gene deletion. Thus, as we discussed before, the design of a tightly controlled inducible expression cassette is also crucial for delivery of peptides such as cytokines which are “toxic” for lactobacilli. The lactobacilli should be induced to express cytokines in the GI tract; and higher or more

frequent dose of the *Lactobacillus* could be given to compensate for the loss of expressing strains in the GI tract.

When the engineered lactobacilli with the expression cassette integrated on the chromosome will eventually be used therapeutically in humans, a biological containment system to avoid the release of the modified lactobacilli into the environment will be required. This system will make bacteria able to grow in the lab but die in the environment, usually controlled by a substance which occurs in low amounts or is absent in the nature. For instance, a thymidylate synthase (*thyA)*-mutant strain cannot survive in a natural environment because thymidine supplement is critical for their life [210, 211], or strains that cannot survive in the absence of unnatural amino acids [212, 213].

To increase the expression level, characteristics of *Lactobacillus* which do not favor cytokine expression, could potentially be eliminated using a powerful genomic editing tool. We have been able to use the single-stranded DNA recombineering (SSDR) assisted with the CRISPR/Cas9 system [194] to knock out the LDH-1 (Lactate dehydrogenase) gene in *L. paracasei* BL23. Furthermore, due to the complexity of the *Lactobacillus* metabolic system, a random selection library is needed to tailor the bacteria for expressing different peptides. Until now, the CRISPR/Cas9 system can only be used as a selection method rather than a real genomic editing tool in prokaryotes, mainly because the bacterium is lacking the NHEJ pathway to repair the double strand break made by CRISPR/Cas9. In the future, an efficient gene knockout library could be established at the genome scale by creating a built-in NHEJ pathway (which has already been demonstrated in *E. coli* [214]) in lactobacilli to facilitate the use of the CRISPR/Cas9 system.

The most surprising result observed in paper I is the significant anti-diabetic effect of *L. paracasei* BL23. *L. paracasei* BL23 is a commonly used laboratory strain for expression of recombinant proteins due to its high transformation rate. The exact origin of the strain is still unclear due to historical reasons [182, 183], and it probably does not form a stable population in the human GI tract. Little work has been performed regarding the probiotic properties of this specific strain [215] but other *L. paracasei /casei* strains have shown versatile probiotic effects including an anti-diabetic effect *in vivo* [216-218]. *L. paracasei* BL23 itself might be used in the future as an alternative treatment for type 2 diabetes. It will also be interesting to study the mechanism involved in the anti-diabetic effect of lactobacilli in order to select better strains or derived products.

Another concern is whether the expression of therapeutic peptides, especially on the surface of lactobacilli, influences the probiotic effect or not. The GLP-1 secreted strain “lost” the antidiabetic effect compare to the wild type strain in paper I. Many studies have shown probiotic activities with killed or lysed lactobacilli [219, 220], indicating that some unique cell wall constituents or cell surface protein may contribute to this effect. The expression of peptides may influence the surface structure, further influencing its colonization and probiotic effect. Considering the safety and efficacy of engineered lactobacilli, all of the strains should

undergo strict quality check (genomic sequencing or *in vitro* probiotic assay including adhesion) to make sure that all the probiotic properties still exist before the clinical trial.

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